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Coral Mucus Is a Hot Spot for Viral Infections

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There is increasing suspicion that viral communities play a pivotal role in maintaining coral health, yet their main ecological traits still remain poorly characterized. In this study, we examined the seasonal distribution and reproduction pathways of viruses inhabiting the mucus of the scleractinians *Fungia repanda* and *Acropora formosa* collected in Nha Trang Bay (Vietnam) during an 11-month survey. The strong coupling between epibiotic viral and bacterial abundance suggested that phages are dominant among coral-associated viral communities. Mucosal viruses also exhibited significant differences in their main features between the two coral species and were also remarkably contrasted with their planktonic counterparts. For example, their abundance (inferred from epifluorescence counts), lytic production rates (KCN incubations), and the proportion of lysogenic cells (mitomycin C inductions) were, respectively, 2.6-, 9.5-, and 2.2-fold higher in mucus than in the surrounding water. Both lytic and lysogenic indicators were tightly coupled with temperature and salinity, suggesting that the life strategy of viral epibionts is strongly dependent upon environmental circumstances. Finally, our results suggest that coral mucus may represent a highly favorable habitat for viral proliferation, promoting the development of both temperate and virulent phages. Here, we discuss how such an optimized viral arsenal could be crucial for coral viability by presumably forging complex links with both symbiotic and adjacent nonsymbiotic microorganisms.

Scleractinian corals are covered by a gel-forming mucus layer, which is of crucial importance in the maintenance of a suitable environment for their survival (1, 2). The polysaccharide-protein composition of this organic matrix constitutes a nutrient-rich ecological niche harboring a great diversity of symbiotic microorganisms for corals (3, 4). Mucosal bacteria, for example, provide corals a part of their nutritive needs (5, 6) and also protection from the surrounding pathogens (7, 8). Such a diverse and multifunctional microbiota is now regarded as a powerful arsenal allowing corals to face environmental stresses by rapid structural adjustments (9–11). Nevertheless, little is known about the adjacent mechanisms involved in such adjustments, the stress threshold triggering this shift, and more generally the factors governing the viability of coral-associated bacteria.

In the ocean's water column, viruses are ubiquitous and represent a prominent agent of bacterial control (12, 13). Recently, they have been demonstrated to be also highly abundant in coral mucus (14, 15), on coral skeleton (16), in the polyp tissues (17), and in the surrounding water (18–20). They have been shown to infect all the microorganisms of the coral holobiont, including the prokaryotic and eukaryotic members (21–24). Then, one might easily anticipate that viruses may also be committed to coral fitness. However, we still lack clear resolution on whether they play beneficial or, conversely, detrimental roles for corals, since they could target either coral symbionts and/or surrounding pathogens. This dual effect was evoked first by van Oppen et al. (25) and later by Vega Thurber and Correa (26). More recently, with the elaboration of the bacteriophage adherence to mucus (BAM) model (27), the role of viral epibiotic communities has reached a further understanding as viruses have been demonstrated, through lytic infections, to actively protect coral surfaces from bacterial pathogen colonization. Recent assays of phage therapy have also revealed that specific bacteriophages could be used as a treatment to cure coral diseases by removing a variety of bacterial infectious agents (28, 29). Although we have now a much clearer comprehension of the role of lytic virulent phages associated to corals, there are still uncertainties regarding many aspects of viral ecology in coral ecosystems. For example, there is no information about the occurrence of lysogenic infections in coral mucus, yet they are widespread in the oceanic water column (30), and they could be potentially crucial for coral health (31). We do not know either if epibiotic viruses can significantly control *in situ* populations of the dinoflagellate *Symbiodinium* since they just have been detected experimentally after UV induction (32) or via genetic evidence (21). Finally, we lack information about their natural occurrence and spatiotemporal dynamics on corals and their ability to effectively create conditions for the disruption of the symbiotic relationship between *Symbiodinium* and the polyp host.

To get better insight into the ecological functions of coral epibiotic viruses and their life cycles and specific interactions with both bacteria and *Symbiodinium*, we carried out a seasonal survey (11 months) in which we performed a microbial inspection of the mucus of two emblematic hard corals of Nha Trang Bay (Vietnam): the free-living *Fungia repanda* and the branching coral *Acropora formosa*.

The study was conducted in the coral reef area of Mot Island (12°10′N, 109°16′E) (Fig. 1). Like most of the coral reefs world-

Received 16 February 2015 **Accepted** 4 June 2015
Accepted manuscript posted online 19 June 2015
Citation Nguyen-Kim H, Bettarel Y, Bouvier T, Bouvier C, Doan-Nhu H, Nguyen-Ngoc L, Nguyen-Thanh T, Tran-Quang H, Brune J. 2015. Coral mucus is a hot spot for viral infections. Appl Environ Microbiol 81:5773–5783. doi:10.1128/AEM.00542-15.
Editor: K. E. Wommack
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doi:10.1128/AEM.00542-15

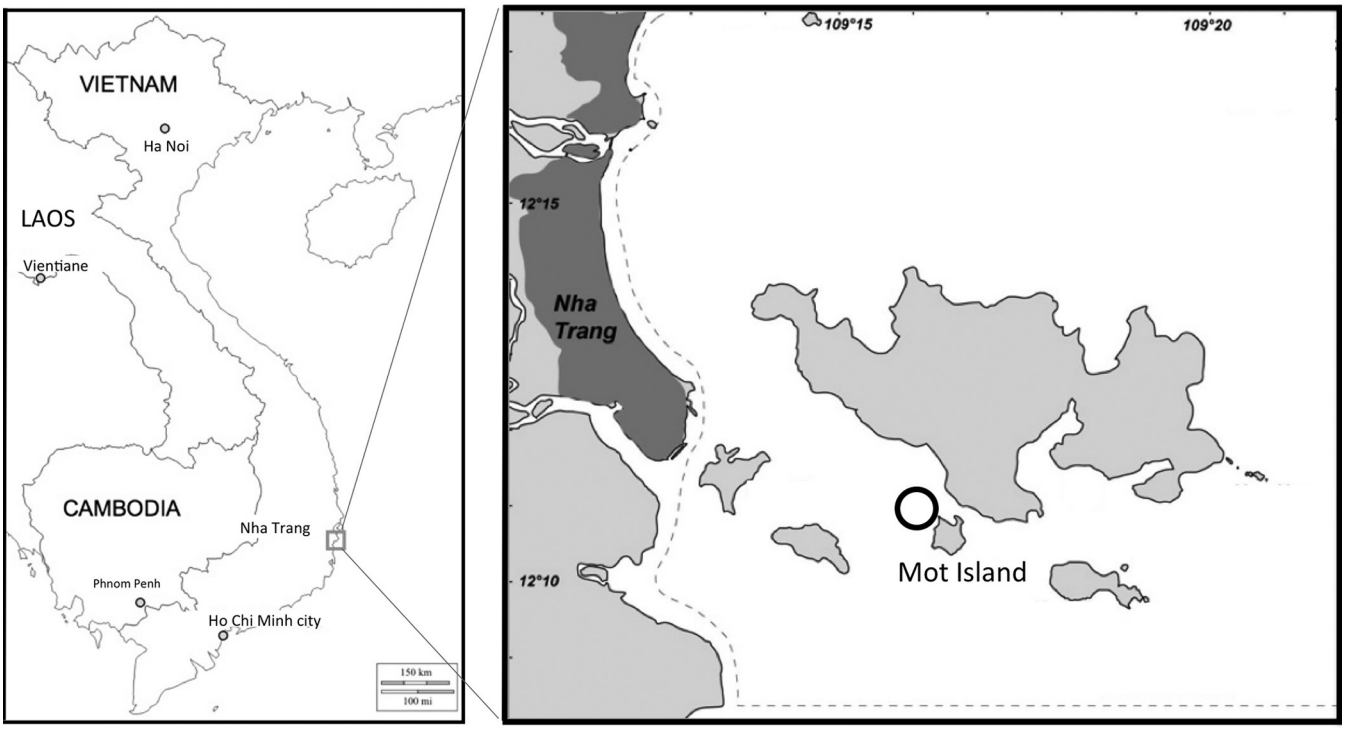

FIG 1 Location of the sampling area (open circle) in Nha Trang Bay, Vietnam.

wide, the Vietnamese ones have been affected by bleaching events, which have become more serious over the past 20 years. Our sampling site is located in the region of Vietnam with the most diverse community of corals, making it a major tourist attraction. According to an International Union for Conservation of Nature (IUCN) report (33), nearly all coral species in this zone have been affected by bleaching over the past few years.

In this study, the specific objectives were (i) to evaluate whether the coral-associated microbiota is influenced by seasonal environmental changes (temperature, salinity, nutrient concentrations), (ii) to determine whether lysogeny is an important phage reproduction pathway in coral mucus, (iii) to study the links between viral traits and the genetic and metabolic diversity of the bacterial associates, and (iv) to examine the *in situ* occurrence of viruses infecting the symbiotic zooxanthellae.

MATERIALS AND METHODS

Sampling site and methods. Two of the dominant scleractinian species of the bay were targeted: the branching coral *Acropora formosa* and the free-living plate coral *Fungia repanda*. The mucus of both corals was collected monthly, at 9 dates from May 2012 to March 2013 (day/month/year: 16/05/2012, 17/06/2012, 18/07/2012, 17/08/2012, 25/09/2012, 06/11/2012, 05/12/2012, 11/01/2013, 13/03/2013). Briefly, duplicate biological samples of the two coral species were collected by scuba diving at depths of 5 to 10 m, in the same area (15 m by 100 m), at each time point. For *A. formosa*, duplicate nubbins of the exact same colony of about 10 m^2 were also sampled. Concurrently, two individuals of *F. repanda* of 15 to 18 cm in diameter were taken in the same area. Mucus was collected by using the desiccation method described in detail elsewhere (34–36). All coral samples were taken out of the water and exposed to air for 1 to 3 min. This stress caused the mucus to be secreted, forming long gel-like threads dripping from the coral surface. The mucus produced in the first 20 s was discarded to prevent contamination and dilution by seawater. The mucus (10 to 12 ml) was then distributed into cryotubes for microbial counts, immediate analysis of the bacterial physiologic and metabolic state, lytic and lysogenic infection rates, transmission electron microscope (TEM) observation, and bacterial genetic and metabolic analyses. Duplicate seawater samples of 15 ml were also collected in the water column before coral sampling, at approximately 1 m above corals, for examination of the parameters described above.

Physicochemical parameters. Duplicate seawater samples were used for chlorophyll *a* (Chl *a*) contents, which were determined by fluorometry after filtration onto Whatman GF/F filters and methanol extraction (37). Salinity and temperature were measured *in situ*, 1 meter above the corals species, by using a conductivity-temperature-depth (CTD) probe (SBE 19+; Sea-Bird Electronics, USA). Precipitation was obtained from the Institute of Meteorology, Hydrology and Climate Change (Nha Trang City, Vietnam).

Counts of microorganisms. Mucus samples were fixed immediately after collection with 0.02-μm filtered formaldehyde (final concentration, 3% [vol/vol]). Fixed mucus was then processed for viral and bacterial extraction by using the potassium citrate method (34; adapted from reference 38). Briefly, a total of 100 μl of fixed mucus was eluted into 900 μl of a 0.02-μm-pore-size-filtered solution of 1% citrate potassium (10 g potassium citrate, 1.44 g $Na_2HPO_4 \cdot 7H_2O$, and 0.24 g KH_2PO_4 per liter). All tubes were then vortexed at a moderate speed for 5 min before particles were stained and enumerated. The numbers of viruses and bacteria contained in each duplicate samples were determined after retention of the particles onto 0.02-μm-pore-size membranes (Anodisc) and staining with SYBR Gold (39). On each slide, 1,000 to 2,000 bacteria and viruses were counted with an Olympus BX53 epifluorescence microscope in 20 fields at a magnification of ×100, under blue light excitation (488 nm). *Symbiodinium* cells, due to their photosynthetic pigments, could also be enumerated on the same slides under the blue light excitation. For planktonic viruses and bacteria, since the extraction procedure was not necessary, we applied only the staining protocol as described above, by using a volume of 300 to 500 μl of seawater.

Fraction of lysogenic bacteria. We used the method of Jiang and Paul (40) to initiate prophage induction in mucosal and planktonic bacteria. Mitomycin C (Sigma-Aldrich) was added to duplicate 5-ml volumes of mucus and water. Duplicate untreated samples served as the control. All samples were formalin fixed (final concentration, 3% [vol/vol]) after being incubated for 10 h, in the dark, at *in situ* temperature. Prophage induction was calculated as the difference in viral abundance (epifluorescence counts, see above) between mitomycin C-treated (V_m) and control incubations (V_c). The fraction of lysogenic bacterial cells (FLC) was calculated as follows: FLC (%) = $100 \times (V_m - V_c)/\mathrm{BS} \times \mathrm{BA}_{to}$, where BS is the burst size (number of viruses per bacterial cell) and BA_{to} is the prokaryote abundance at the start of the experiment, i.e., before adding mitomycin C (41, 42). Since no burst size from infected mucus-associated bacteria has been published so far, we used a burst size of 24, which is an average value calculated from the range for different studied environments (43).

Prior to carrying out the quantification of the fraction of lysogenic cells, we performed a preliminary experiment to determine the most efficient concentration of mitomycin C and incubation time. Three different concentrations of mitomycin C were tested: 1, 3, and 5 $\mu g\ ml^{-1}$ over a 30-hour incubation, with samplings at 5-hour intervals. The results showed maximum induction after 10 h by using the concentration of 1 $mg\ ml^{-1}$ (data not shown).

Viral lytic production. The decay, i.e., the decrease in the viral concentration over time, was recorded after inhibition of new viral lytic production (VP) by the addition of potassium cyanide (KCN; final concentration, 2 mM) in both mucus and water samples (44, 45). All incubations for decay experiments were performed in duplicate at *in situ* temperature, for 12 h (46). Incubations were stopped after addition of formaldehyde (final concentration, 3%). Viral abundance was determined in KCN-treated and untreated water and coral mucus, by using SYBR Gold and epifluorescence microscopy (see above). The difference between the abundance of viruses with and without KCN allows the estimation of VP.

TEM observation of viral infection in bacteria and *Symbiodinium* cells. Duplicate 3-ml mucus samples were fixed in a solution of 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and stored overnight at 4°C. To make the pellet, samples were then centrifuged for 15 min at 3,000 rpm and after that washed three times in 0.1 M cacodylate buffer for 15 min and postfixed in 1% osmium tetroxide for 1 h at room temperature. Samples were then rinsed twice in 0.1 M cacodylate buffer (10 min), dehydrated in an ethanol series (50%, 70%, 90%, and 100%) and propylene oxide (10 min per ethanol concentration), and embedded in Epon 812. Thin 60- to 80-nm sections were obtained by ultramicrotome (Leica, UC6), using collodion-coated copper grids (300 mesh). The sections were double stained for 5 min with 5% uranyl acetate and 10 min with lead citrate and examined at 80 kV by transmission electron microscopy (JEOL JEM 1010) to distinguish between visibly infected and uninfected bacteria and *Symbiodinium* (47, 48). At least 100 bacterial cells and 30 *Symbiodinium* organisms were inspected per grid.

Bacterial respiring activity. The proportion of respiring bacteria that have high rates of metabolism was determined using 5-cyano-2,3-ditolyltetrazolium chloride (CTC), an indicator of the respiratory electron transport system activity (49). A stock solution of 50 mmol $liter^{-1}$ CTC (tebubio SAS) was prepared daily, filtered through 0.02-μm filters, and kept in the dark at 4°C until use. A CTC stock solution was then added to 0.45 ml of both duplicate fresh mucus and water samples (5 mmol $liter^{-1)}$ and incubated for 1.5 h at room temperature in the dark. Formaldehyde (final CTC concentration, 3%) was used to stop the CTC reaction. Samples were flash frozen in liquid nitrogen and stored in a −80°C freezer until flow cytometer (FCM) analysis. The red fluorescence of CTC (FL3) and the light scatter (side scatter [SSC]) were used to discriminate the CTC+ cells from other cells or weak fluorescent particles (15). The percentage of CTC+ cells, based on triplicate analyses, was calculated relative to the total bacterial counts obtained by epifluorescence microscopy.

DGGE analyses of bacterial community structure. The genetic diversity of eubacterial communities in both mucus and water samples was estimated for the different months sampled (except August for *A. formosa*) by using the denaturing gradient gel electrophoresis (DGGE) fingerprinting technique. Water (500 ml) and mucus samples (500 μl resuspended in 1 ml of 9‰ NaCl buffer) were filtered on 0.2-μm polycarbonate filters (Whatman) for total extraction of DNA and stored in liquid nitrogen before analysis. Only one of the two sets of replicates (including mucus and water samples) was treated. The DNA sequences were then subjected to touchdown PCR using primers 341F-GC and 519R (50), which target the V3 region of bacterial 16S rRNA gene (178 bp). PCR was carried out with 10 ng of extracted DNA and the kit PuRe Taq Ready-To-Go PCR beads (GE Healthcare) using the PCR touchdown program (51) with a Mastercycler (Eppendorf). Thirty-five cycles of amplification were done starting with a 93°C initial denaturation of the double-stranded DNAs (dsDNAs), followed by a second denaturation phase at 92°C and the annealing step, which was done at the high temperature of 71°C, minimizing unspecific primer binding. After each cycle, the temperature was lowered by 0.5°C until reaching the touchdown temperature of 61°C, keeping that temperature for the last 15 cycles. PCR products were verified in 1.5% (wt/vol) agarose gel using the SYBR Green I nucleic acid gel stain (1:10,000 dilution; Molecular Probes) and quantified on NanoDrop ND 1000 (ThermoScientific). PCR samples were loaded onto 8% (wt/vol) polyacrylamide gels made with a denaturing gradient ranging from 35 to 65% (100% denaturant contains 7 M urea and 40% formamide). For visualization of the bacterial community structure, the gels were run on an Ingeny U-Phor system (Ingeny) in 0.5× Tris-acetate-EDTA (TAE) buffer (Euromedex) at 60°C with a constant voltage of 80 V for 18 h. The DNA was then stained for 10 min with 3 μl of 10,000× SYBR Green I (Life Technologies) diluted in 15 ml 0.5× TAE. DNA bands were visualized on a UV transillumination table with the imaging system GelDoc XR (Bio-Rad) and analyzed using fingerprint and gel analysis Quantity One software (Bio-Rad). First, each sample lane was identified and the background subtraction was applied. Each single band in each lane was then normalized to a reference standard on each gel. Finally, each band was identified and quantified. After generating a band-matching table, we obtained the binary matrix of relative intensity for all the detected bands. This data set was used to calculate a distance matrix with the Bray-Curtis dissimilarity index ($\mathrm{BC}_{\mathrm{DGGE}}$), which subsequently was used for an ordination analysis, i.e., principal coordinate analysis (PCoA) (see "Data analysis" below).

Biolog analysis of bacterial metabolic capacities. The Biolog microtiter EcoPlate was used to assess the community level physiological profiles (CLPP) of bacterial communities following carbon sources utilization and can be applied to study the metabolic diversity of coral-associated bacterial communities (52). The limitation of this approach has been detailed elsewhere (53). The replicates used were those chosen for the DGGE analysis. One milliliter of each unfixed mucus sample was diluted in 19 ml of bacteria-free 0.02-μm-filtered seawater and loaded to the EcoPlate (140 μl per well), which was then incubated aerobically in the dark at *in situ* temperature. One plate was used per coral species. For seawater samples, 140 μl was directly inoculated per well. These plates consist of 96 microwells, containing 31 different carbon sources and one control (no source of carbon) in triplicate. Mineralization of the carbon substrate was revealed by the development of a purple color, due to the reduction of tetrazolium dye contained in each well. The color development assessed by the optical density (OD) of each plate was read at 24-h intervals, up to 168 h after inoculation, using a SmartSpec3000 spectrophotometer (Bio-Rad) at the wavelength of 595 nm (absorbance peak of tetrazolium).

The average well color development (AWCD) at each reading time was determined using the following formula: $\mathrm{AWCD}_{(t)} = [\Sigma(C_{(t)}\text{-}R_{(t)})]/31$, where t is the time of incubation, C is the mean color production of each substrate (standardized OD measurement) at time t, and R is the absorbance value of the control well (54). Subsequently, based on the examination of the kinetic curves of AWCD, we chose to use the OD

TABLE 1 Physicochemical parameters in Mot Island

Parameter (unit)	2012								2013		
	May	June	July	August	September	October	November	December	January	February	March
Temp (°C)	29.5	27.8	29.4	27.8	29.7		28.2	27.5	25.2		26.7
Salinity (‰)	32.8	33.6	34.3	33.5	30.8		32.2	32.3	32.8		33.0
Chlorophyll *a* concn (μg liter^{-1})	0.37	0.59	0.33	0.33	0.38		0.15	0.56	0.75		0.26
Amt of precipitation (mm)	92.4	24.3	151	29.6	444.6	104.3	370.3	38.6	63.6	34.8	15.2

obtained after 168 h of incubation, i.e., when the stationary phase was reached. The data at this chosen time were standardized by the following formula: $(C - R)$/AWCD. Finally, the standardized values that were <0 and >2 were truncated before the data were subjected to statistical analysis. A qualitative matrix (presence/absence) was subsequently constructed and used to calculate a distance matrix using the Bray-Curtis dissimilarity index (BC_{CLPP}). A principal coordinate analysis was performed based on this distance matrix (see "Data analysis" below).

Data analysis. A nonparametric Kruskal-Wallis test was performed using JMP 9.0 software from the SAS Institute test to compare differences between samples in all studied variables. Each piece of data from each date of sampling was considered to be one replicate. To explore the strength of the relationships among the temporal variations of two studied coral mucus samples, the Pearson correlation coefficient was calculated by using the multivariate platform option in the software JMP 9.0. This correlation coefficient was also used to determine the relationships between different studied parameters.

In order to visualize the variations in bacterial genetic and metabolic diversity between samples through the studied period, a PCoA was applied, using Bray-Curtis dissimilarity matrices as inputs. To further observe the differences as well as to compare the variability between samples, a centroid or average plotting position for each category of sample group was determined and plotted onto the same ordination PCoA graph. All the variables were then connected with the centroid of the same category. The distances from the centroid to each variable were calculated and provided three data sets of distances. The differences in the distance data set, representing the variability of each sample during the survey, were then plotted as boxplots and tested for differences by using the Kruskal-Wallis test. The analysis and calculation were done using R software version 2.1 with the ade4 and vegan packages.

The Mantel test with the permutation of 999 was performed using R software version 2.1 with the package ade4 to evaluate the possible relation between the two Bray-Curtis dissimilarity matrices determined for the genetic and metabolic bacterial diversity. The same test was also used to evaluate whether all the studied parameters were related to shifts in genetic and metabolic bacterial diversity in all the samples (Bray-Curtis matrices).

RESULTS

Physicochemical parameters. During the survey (May 2012 to March 2013), water temperature, chlorophyll *a*, and salinity showed weak seasonal variations (Table 1). On average, the water temperature was 28.0°C, with the highest temperature recorded in September (29.7°C) and the lowest in January (25.2°C). During the survey, no trace of coral bleaching or injuries was observed in any of the target species. Precipitation levels (minimum to maximum, 15.2 to 444.6 mm) and salinity (minimum to maximum, 30.8 to 34.3) exhibited high temporal variability (Table 1). The highest rainfalls were observed in September and November (444.6 and 370.3 mm, respectively), during which times salinity was at minimum values (30.8 and 32.2, respectively). The chlorophyll *a* concentration in the sampling site ranged between 0.15 μg liter^{-1} in November and 0.75 μg liter^{-1} in January. Chlorophyll *a* concentration did not show any significant correlation with any other biotic or abiotic parameters (Table 2).

Abundance of viruses, bacteria, and *Symbiodinium*. On average, from all the different samples collected over this seasonal study, the abundance of epibiotic viruses ($\bar{x} = 3.9 \times 10^7$ viruses ml^{-1}) was significantly higher than that of their planktonic counterparts ($\bar{x} = 1.5 \times 10^7$ viruses ml^{-1}) (Kruskal-Wallis, $P < 0.01$, $n = 9$). Viruses were significantly more abundant in the mucus of *A. formosa* ($\bar{x} = 5.4 \times 10^7$ viruses ml^{-1}) than in that of *F. repanda* ($\bar{x} = 2.3 \times 10^7$ viruses ml^{-1}) (Table 3; Fig. 2A).

Bacterial concentrations were also significantly higher in mucus ($\bar{x} = 6.8 \times 10^6$ cells ml^{-1}; Kruskal-Wallis, $P < 0.01$) than in water samples ($\bar{x} = 3.5 \times 10^6$ cells ml^{-1}). There was also a strong interspecies variability in the concentration of coral-associated bacterial communities, with almost 2-fold more cells in the mucus

TABLE 2 Pearson correlation coefficients between basic parameters in water and mucus samples[a]

Parameter	Water/mucus Pearson correlation coefficients									
	VIR	BAC	SYMB	CTC	FLC	VP	Temp	Sal	Chl *a*	Rain
VIR	1									
BAC	**0.82/0.78**	1								
SYMB	NA/0.03	NA/−0.21	1							
CTC	0.21/−0.59	−0.13/−0.56	NA/−0.26	1						
FLC	**0.84**/0.27	**0.80**/−0.25	NA/−0.58	−0.11/0.20	1					
VP	0.33/0.14	0.14/−0.40	NA/−0.21	0.13/0.04	0.05/**0.81**	1				
Temp	0.33/0.29	0.41/0.03	NA/0.05	−0.21/−0.11	**0.82/0.82**	0.75/**0.87**	1			
Sal	**−0.87**/0.36	**−0.69**/0.35	NA/0.29	−0.29/0.10	**−0.90/−0.97**	−0.73/**−0.98**	−0.15	1		
Chl *a*	0.0/−0.51	0.11/−0.45	NA/0.02	−0.01/0.46	−0.35/−0.17	−0.04/−0.09	−0.52	0.05	1	
Rain	**0.78**/0.08	**0.64**/−0.06	NA/−0.40	0.49/−0.11	**0.85/0.81**	0.42/**0.76**	0.52	**−0.68**	−0.43	1

[a] Significant relationships are shown in bold ($P < 0.05$). NA, not available (i.e., no *Symbiodinium* cells were found in seawater, so the Pearson correlation coefficient could not be calculated).

TABLE 3 Values for the different parameters measured in coral mucus and water samples[a]

Sample	Mean value (range)						
	VIR (10^7 ml^{-1})	BAC (10^6 ml^{-1})	SYMB (10^5 ml^{-1})	VBR	FLC (%)	VP (10^6 ml^{-1} h^{-1})	CTC+ (%)
Water	1.5 (0.7–3.7)	3.5 (1.9–7.8)	0.0	4.3 (2.2–8.3)	3.8 (0–12.2)	0.2 (0.1–0.4)	11.3 (0.5–35.0)
F. repanda mucus	2.3 (1.3–3.9)	4.5 (2.4–9.6)	0.1 (0–1.4)	5.8 (2.7–11.7)	8.4 (1.1–25.4)	1.1 (0.4–2.2)	23.6 (2.7–67.3)
A. formosa mucus	5.4 (2.4–12.2)	9.2 (2.5–16.7)	1.3 (0.5–2.6)	6.8 (4.9–10.8)	8.5 (3.3–20.0)	2.6 (0.6–4.1)	45.8 (4.1–80.7)

[a] VIR, viral abundance (no. of viruses); BAC, bacterial abundance (no. of bacterial cells); SYMB, no. of *Symbiodinium* cells; VBR, virus-to-bacterium ratio; CTC+, fraction of CTC-positive cells; FLC, fraction of lysogenic cells; VP, viral production (no. of viruses).

of *A. formosa* ($\bar{x} = 9.2 \times 10^6$ cells ml^{-1}) than in that of *F. repanda* ($\bar{x} = 4.5 \times 10^6$ cells ml^{-1}; $P < 0.01$) (Table 3). Seasonally, the highest concentrations of viral and bacterial epibionts were recorded in July, when salinity reached its maximum value. Conversely, these communities did not show any clear temporal variability in the water column (Fig. 2). The temporal dynamics of bacterial abundance of the two coral species were similar ($r = 0.70$, $P < 0.05$, $n = 9$) (Fig. 2B). Overall, there was a significant correlation between viral and bacterial abundances in both mucus samples ($r = 0.78$, $P < 0.01$, $n = 18$) and water samples ($r = 0.82$, $P < 0.01$, $n = 18$) (Table 2). The virus-to-bacteria ratio (VBR) averaged 6.3 in coral mucus and 4.4 in water, the difference being significant (Kruskal-Wallis, $P < 0.01$) (Table 3; Fig. 2C). Significant differences were also recorded between the VBR measured in the mucus of *A. formosa* ($\bar{x} = 6.8$) and that of *F. repanda* ($\bar{x} = 5.8$) (Kruskal-Wallis, $P < 0.05$).

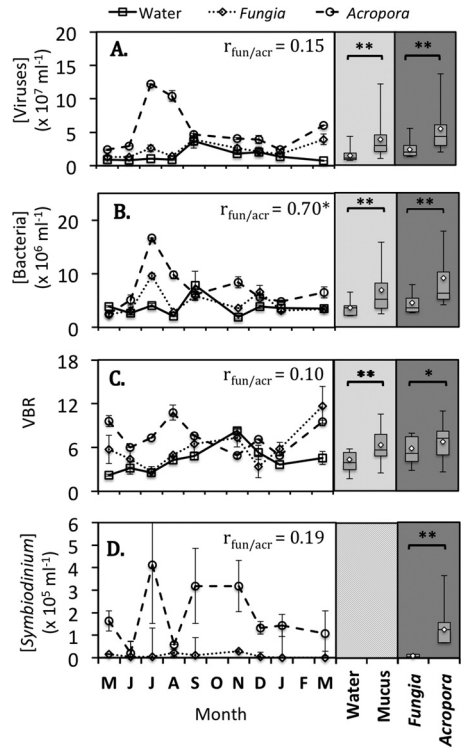

FIG 2 Abundances of viruses (A), bacteria (B), and *Symbiodinium* (D) and VBR values (C) in the two coral mucus and water samples. $r_{fun/acr}$, Pearson correlation coefficient (r) of each parameter between the two coral species *Fungia repanda* (fun) and *Acropora formosa* (acr); *, significant correlation. Error bars represent 1 standard deviation from the mean ($n_{mucus} = 36$, $n_{water} = 18$). The boxplots on the right side of each graph show the comparison of the average values between water and mucus (for both coral species) (light gray plot) and *A. formosa* and *F. repanda* (dark gray). Significant differences among samples (Kruskal-Wallis test: *, $P < 0.05$; **, $P < 0.01$) are indicated by brackets. Boxplots represent the 50th (median), 75th, and 25th percentiles. Diamonds represent mean values of mucus ($n = 36$) and water ($n = 18$) samples.

No *Symbiodinium* cells could be detected in any of the water samples. The average concentration of *Symbiodinium* was 14-fold higher in the mucus of *A. formosa* ($\bar{x} = 1.3 \times 10^5$ cells ml^{-1}) than in that of *F. repanda* ($\bar{x} = 9 \times 10^3$ cells ml^{-1}) (Kruskal-Wallis, $P < 0.01$) (Table 3; Fig. 2D). No significant correlations were found between the abundance of *Symbiodinium* and that of viruses or bacteria (Table 2).

In coral mucus samples, the concentrations of viruses, bacteria, or *Symbiodinium* were not correlated with any of the environmental parameters recorded (i.e., temperature, salinity, chlorophyll *a*, or precipitation). Conversely, in the water column, the levels of salinity and rainfall were strongly correlated with the abundance of planktonic viruses and bacteria (Table 2).

TEM observations of thin sections of mucosal bacteria and *Symbiodinium*. Among the more than 300 *Symbiodinium* cells that were inspected by transmission electron microscopy (TEM) for each coral species, none of them showed any visible sign of viral infection in the nucleoplasm or the cytoplasm (Fig. 3A). Almost identically, of the more than 3,000 examined mucosal bacterial cells only 1 cell revealed the presence of intracellular viruses (Fig. 3D) while all the other cells were seemingly uninfected (Fig. 3B). Given the methodological difficulties encountered with the TEM inspection of mucosal bacteria and the quasi absence of visibly infected cells, we thus decided to switch to the KCN approach from September onward to estimate the lytic activity of coral-associated viral communities (see below).

Lytic infections. The sodium cyanide (KCN) method was used to estimate viral lytic production (VP) from September 2012 to March 2013. On average, VP was significantly higher in the mucus ($\bar{x} = 1.9 \times 10^6$ viruses ml^{-1} h^{-1}) than in the surrounding water ($\bar{x} = 0.2 \times 10^6$ viruses ml^{-1} h^{-1}; Kruskal-Wallis, $P < 0.01$, $n = 5$) (Fig. 4, lower panel). The mucus of *A. formosa* exhibited a significantly greater VP than that of *F. repanda* (Kruskal-Wallis, $P < 0.05$, $n = 5$).

Lysogenic infections. Concurrently with viral lytic production, the frequency of lysogenic cells (FLC) was also estimated from September 2012 to March 2013 by using a chemical inducer, mitomycin C (41). On average, FLC was more than two times higher in the mucus ($\bar{x} = 8.5\%$) than in the water ($\bar{x} = 3.8\%$), and the difference was statistically significant (Kruskal-Wallis, $P < 0.05$, $n = 5$) (Table 2; Fig. 4, upper panel). The temporal variations of FLC followed the same trend in mucus and water, characterized by a continuous decrease from September to December, followed by a relative stability of the values until the end of the survey (March). No significant differences could be found between the two scleractinian species

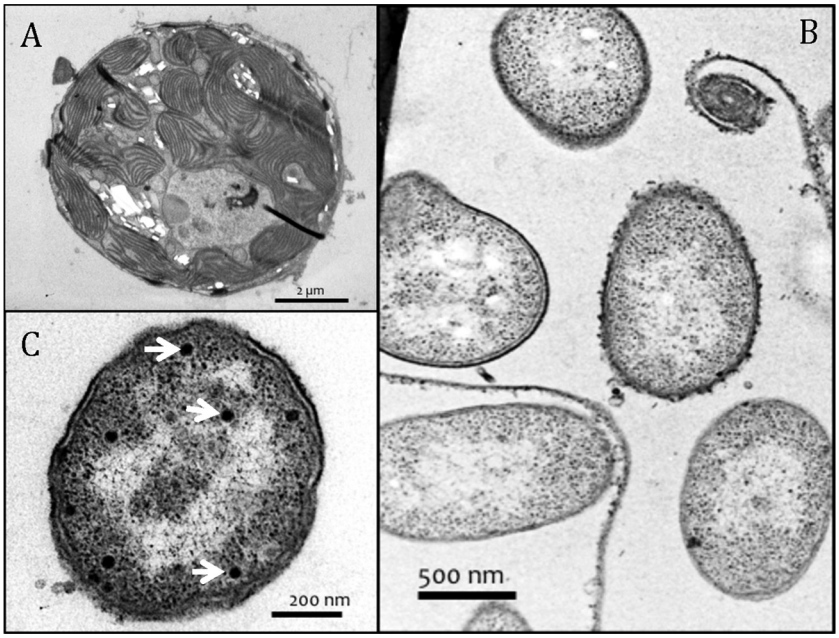

FIG 3 Transmission electronic microscopy (TEM) images of thin sections of a healthy free-living *Symbiodinium* cell in the mucus of *F. repanda* (A), mucosal bacteria showing no traces of viral infection (B), and a virally infected bacterial cell showing several intracellular viruses (white arrows) (C).

(Fig. 4, upper panel). In the water samples, we observed positive and significant correlations between FLC and both viral and bacterial abundances. However, this was not the case in coral mucus samples. Interestingly, FLCs measured in both compartments were significantly correlated with temperature, salinity, and precipitation levels (Table 3). A positive and significant correlation was found between FLC and VP in the mucus ($r = 0.81$, $P < 0.01$, $n = 5$) but not in water (Table 3).

Physiological state of prokaryotic community. The fluorogenic tetrazolium dye 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC) was used to determine the actual proportion of bacteria that were actively engaged in the respiratory process (50). As for most of the studied parameters, the mean proportion of active respiring bacteria (CTC+ cells) was substantially and significantly higher in coral mucus ($\bar{x} = 34.7\%$) than in the overlaying water ($\bar{x} = 11.3\%$) (Kruskal-Wallis, $P < 0.01$, $n = 9$) (Table 2; Fig. 5). There was also an interspecific significant difference between the amounts of CTC recorded in the mucus of *A. formosa* (mean, 45.8%) and in that of *F. repanda* (mean, 23.6%).

The temporal dynamics of the proportion of active cells observed for water samples was dissimilar to that of both mucus samples (Fig. 5). However, the trend was comparable for the two coral species ($r = 0.63$, $P < 0.05$, $n = 9$) and showed strong fluctuations throughout the survey (Fig. 5). No significant correlation could be detected between the percentages of CTC+ cells with all of the studied variables during the survey, regardless of whether the samples were mucus or water.

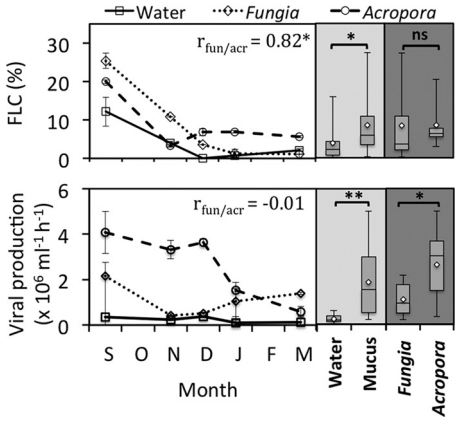

FIG 4 Fraction of lysogenic cells (upper panel) and viral lytic production (lower panel) in both coral mucus and water samples. $r_{fun/acr}$, Pearson correlation coefficient of each parameter between the two coral species; *, significant correlation. The gray boxplot on the right side of each graph shows the comparison of the average values of 3 samples. Significant differences among samples (Kruskal-Wallis: *, $P < 0.05$; **, $P < 0.01$) are indicated by brackets.

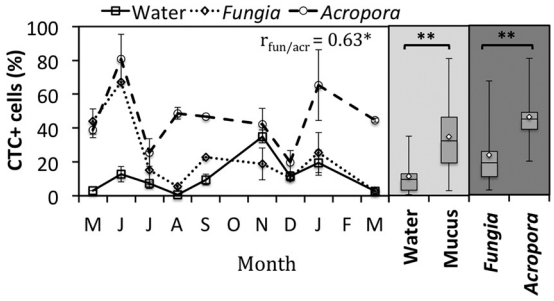

FIG 5 Percentages of CTC+ cells in the both coral mucus and water samples. $r_{fun/acr}$, Pearson correlation coefficient of each parameter between the two coral species; *, significant correlation. The boxplots on the right side of each graph show the comparison of the average values between water and mucus (light gray plot) and between *A. formosa* and *F. repanda* (dark gray). Significant differences among samples (Kruskal-Wallis: **, $P < 0.01$) are indicated by brackets.

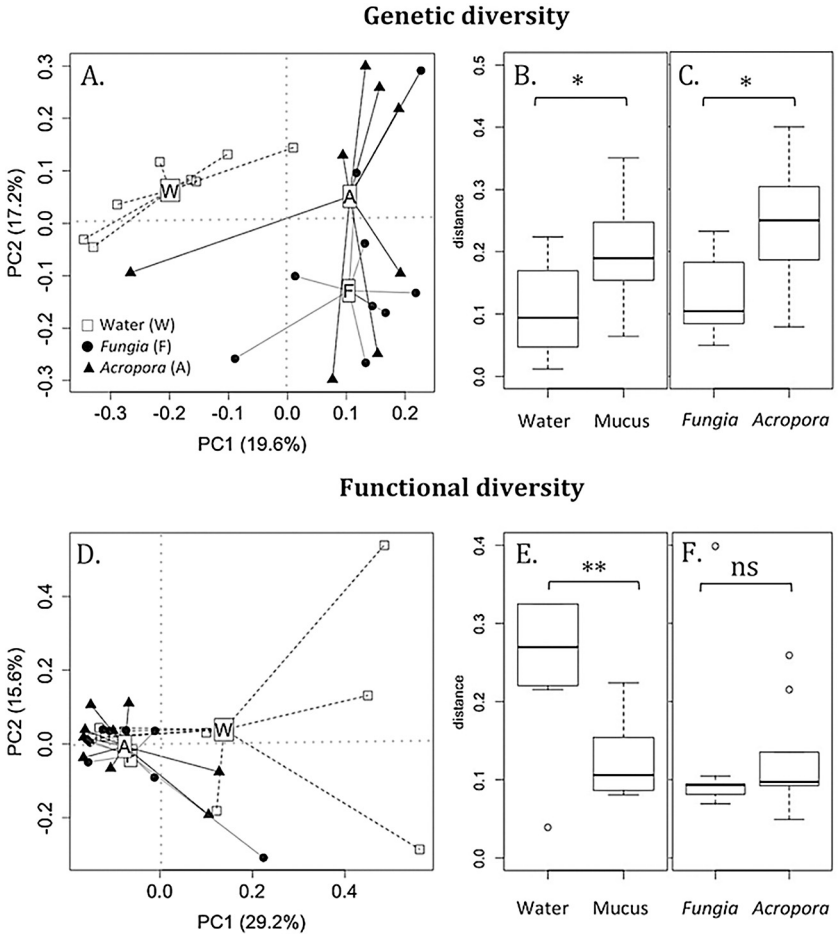

FIG 6 (A to C) Principal component analysis (PCoA) obtained with the Bray-Curtis dissimilarity matrix calculated from bacterial DGGE data. Principal coordinate 1 (PC1) versus principal coordinate 2 (PC2) are represented (A). Boxplots on the right side of the PCoA plots indicate the comparison of the variability in bacterial genetics between water and mucus (B) and between *A. formosa* and *F. repanda* (C). (D to F) Also shown is PCoA of data (D) with the comparison of the variability in bacterial genetics between water and mucus (E) and between *A. formosa* and *F. repanda* (F). Significant differences among samples (Kruskal-Wallis: *, $P < 0.05$; **, $P < 0.01$) are indicated by brackets.

DGGE assessment of bacterial community structure. The bacterial community structure (BCS) obtained by denaturing gel gradient electrophoresis (DGGE) in mucus and water samples was clustered into different groups by applying a principal coordinates analysis (PCoA) based on the Bray-Curtis dissimilarity index (BC_{DGGE}) (Fig. 6A). The planktonic and mucosal communities were, in most cases, separated by the first principal coordinate (PC) (19.6% of the variation). The two coral species were mainly clustered close to each other, along the PC2 (17.2% of the variation). Figure 6B shows the average distance values calculated from the distances drawn from the centroid to each variable (month) of each sample. This distance relates to the temporal variability of the BCS, which was significantly higher in mucus than in water (Kruskal-Wallis, $P < 0.05$) (Fig. 6B). The BCS of *A. formosa* showed a higher seasonal variability than that of *F. repanda* (Kruskal-Wallis, $P < 0.05$) (Fig. 6B).

The Mantel test did not reveal any significant correlations between BC_{DGGE} with most of the studied parameters, except a positive correlation with the viral abundance in water and the mucus of *F. repanda* (Table 4).

Metabolic diversity of bacterial epibionts. The comparison of the metabolic diversity or community level physiological profiles (CLPP) of bacterial communities in the different samples was estimated with the Biolog EcoPlate, followed by a principal coordinates analysis (PCoA) of the Bray-Curtis distance (BC_{CLPP}). As was the case for the bacterial community structure measured with DGGE (see above), both corals were clearly separated from water samples (Fig. 6D). The metabolic diversities of *F. repanda* and *A. formosa* were highly comparable, as illustrated by the clustering of the two groups. However, in contrast to what was observed for the genetic diversity, the metabolic diversity of the planktonic communities showed larger seasonal variations than that of their mucosal counterparts (Kruskal-Wallis, $P < 0.01$) (Fig. 6B). Finally, no significant differences were found between the two coral species (Fig. 6C).

The Mantel tests showed only the links of viruses to the metabolic diversity of the bacterial communities in water ($r = 0.50$, $P < 0.05$) and in the mucus of *F. repanda* ($r = 0.44$, $P < 0.05$) (Table 4). Among all the parameters, only rainfall was found to have influence on the metabolism of the planktonic bacterial community ($r = 0.65$, $P = 0.03$).

TABLE 4 Summary of Mantel correlation tests[a]

Dissimilarity index[b] and parameter	Water		*Fungia*		*Acropora*	
	r	*P*	*r*	*P*	*r*	*P*
BC_{DGGE} versus:						
VIR	0.54	**0.04**	0.39	**0.05**	0.23	0.20
BAC	0.47	0.09	0.05	0.43	0.00	0.53
CTC	−0.22	0.80	−0.26	0.85	−0.23	0.82
FLC	0.79	0.16	−0.03	0.53	0.21	0.39
VP	0.1	0.29	0.34	0.13	0.35	0.15
TEMP	0.28	0.14	−0.33	0.94	0.3	0.07
SAL	0.4	0.08	0.24	0.13	0.08	0.4
Chl *a*	−0.23	0.84	−0.18	0.80	−0.06	0.60
PREC	0.26	0.17	0.14	0.25	−0.06	0.50
BC_{CLPP} versus:						
VIR	0.50	**0.05**	0.44	**0.04**	−0.21	0.76
BAC	0.48	0.09	−0.14	0.59	−0.35	0.97
CTC	0.36	0.18	−0.08	0.48	0.02	0.65
FLC	0.48	0.21	−0.26	0.64	−0.25	0.49
VP	−0.48	0.99	−0.43	0.83	0.09	0.54
TEMP	−0.07	0.49	0.24	0.19	0.23	0.21
SAL	0.35	0.12	−0.38	0.1	−0.2	−0.69
Chla	0.06	0.38	−0.04	0.52	0.49	0.06
PREC	0.64	**0.02**	−0.18	0.68	−0.19	0.67
BC_{DGGE} versus BC_{CLPP}	0.35	0.10	0.04	0.43	−0.012	0.48

[a] Significant differences ($P < 0.05$) are shown in bold.
[b] BC_{DGGE}, Bray-Curtis dissimilarity index for the community composition estimated by DGGE; BC_{CLPP}, Bray-Curtis dissimilarity index for the community level physiological profiles (CLPP).

DISCUSSION

Phages are highly abundant in coral mucus. Viruses were by far the most abundant biological entities found in the mucus of both *A. formosa* and *F. repanda*. By comparison, the average concentrations of epibiotic bacteria and *Symbiodinium* were, respectively, 6- and 600-fold lower. These results are in line with previous reports showing that viruses are quantitatively dominant within the coral holobiont (14, 15, 22). As was the case in the water column, we observed a positive and highly significant correlation between viral and bacterial abundance in mucus, suggesting that the majority of viral epibionts of corals were actually phages. Although there is genetic and morphological evidence for the presence of phages in coral mucus (14, 22), their quantitative predominance within the viral assemblage was uncertain, until now. Surprisingly, the TEM examination of thin sections of mucosal bacteria showed the presence of intracellular viruses in only one out of several thousand cells (Fig. 3C). The rare occurrence of visibly infected cells in this study could be due to a particularly short rise phase in the mucosal phage life cycle, during which virions are visible within the cell ultrastructure. Alternatively, the centrifugation step of the TEM protocol may also have caused the disruption of virally infected cells. To cope with this last potential methodological bias, we concurrently estimated viral lytic activity with the KCN method, which is frequently used with planktonic and benthic cells (47, 55, 56). Given the high viscosity and the small volumes of mucus available (<10 ml), we could not apply the viral reduction approach to estimate viral lytic production rates (57). Then, the KCN method, a cellular poison used to stop viral production, emerged as the best compromise (46, 55, 56). The results obtained with this method revealed that lytic infections are actually widespread in coral mucus. The production rates of viral epibionts were even more than 9-fold higher than those in the overlaying water. This result, together with the tight coupling between viral and bacterial concentrations described above, seems to suggest the prevalence of phages over viruses of eukaryotes within the community of viral epibionts.

In this study, epibiotic bacteria were also much more active ($\bar{x}$ = 34.7% CTC+ cells) than planktonic cells ($\bar{x}$ = 11.3% CTC+ cells), which corroborates previous reports showing that coral associates are comprised of active and fast-growing cells (15, 58, 59). However, no significant correlation was found between the proportion of CTC+ cells and viral abundance or production, suggesting that viruses are not necessarily produced by the more active fraction of epibiotic cells. Alternatively, there may be a proportion of CTC+ cells that are only rarely used as viral hosts, although the viruses are still occupying mainly the CTC+ fraction of the epibiotic cells. Ascertaining whether or not these active cells represent key symbionts for corals still needs further investigation.

Lysogeny in coral mucus. Lysogeny has long been regarded as an important viral strategy in oceanic waters, where environmental conditions are unfavorable for the growth of their hosts and thus their own proliferation (30, 60). Lysogenic infections then typically prevail in oligotrophic, cold, nutrient-poor environments (61, 62). In this study, we estimated the proportion of mucosal bacteria under the lysogenic stage of infection, both in water and in mucus samples. Possible methodological bias may exist, as we conducted *ex vivo* incubations, and the results may thus differ from what occurs under *in situ* conditions. Also, for the reasons mentioned above, we could not use the viral reduction approach (57) to prevent new viral infection from occurring during the incubations with coral mucus. Therefore, our results should be likely considered underestimates of the proportion of lysogens in

our samples. Although not all lysogens are inducible by mitomycin C and there could be high variability from sample to sample (40), this approach still remains the most robust and widely used, as there is no other reliable alternative (42). Unexpectedly, FLC was more than twice as high in coral mucus ($\bar{x}$ = 8.5%) as what was observed in the overlying water ($\bar{x}$ = 3.8%). These results were surprising, as coral mucus is of high nutritive value for bacterial symbionts (1). Yet, lysogeny could be also potentially of great benefit for mucosal symbiotic bacteria, since such latent infections may also paradoxically provide the lysogens protection from other virulent phages (30), thus allowing them to best achieve their main functions (i.e., nutrition and defense) for corals. The beneficial effects of lysogeny for coral animals may occur only during times of stable environmental conditions, whereas conversely, environmental perturbations (including strong variations in temperature, salinity, nutrients, pollutants, etc.) might trigger prophage induction, the subsequent lysis of the symbionts, and finally the onset of coral diseases (31).

We also found a positive and significant correlation between the proportion of lysogens (FLC) and viral lytic production (VP) in the mucus, suggesting that both lysogenic and lytic life cycles seem to coexist, and this coexistence is probably crucial for corals in ways that still remain to be elucidated. In the mucus, both viral lytic production and the proportion of lysogens were tightly coupled with environmental conditions (i.e., temperature and salinity), suggesting that the life strategy of viral epibionts of corals, in some yet-unknown ways, could be dependent upon climatic forces. These results provide some support to the recent hypothesis that viruses, by using environmentally driven infection strategies, might regulate, circumstantially, either coral bacterial symbionts or surrounding pathogens (31). Indeed, it has been suggested that when seawater temperature is stable, phage lysogenic and lytic infections may contribute to coral fitness by providing immunity to bacterial symbionts against other viral infection and lytic protection against opportunist surrounding cells (pathogenic or not), respectively (see the BAM model in reference 27). Conversely, in times of thermal anomalies, the resulting induction of lysogens together with the thermal alteration of lytic phages may impair the bacterial symbionts and favor pathogen colonization, thus ultimately hastening coral decline (31).

Viral traits and their ecological links with bacterial community composition and metabolic capacities. DGGE analyses revealed contrasted profiles between mucosal and planktonic assemblages. These results are not surprising since coral mucus has been long shown to represent a selective medium for specific bacterial associates genetically and functionally distinct from planktonic communities (2, 58, 63, 64). The PCoA plot based on the Bray-Curtis dissimilarity index also showed a greater seasonal variability in the community structure of epibiotic than in that of the surrounding planktonic cells. Such substantial fluctuations seem to confirm the highly plastic nature of coral associates and their ability to efficiently adapt to environmental stress by altering their structure (65, 66). This genetic versatility might represent for corals a way to adjust and face the seasonal shifts in, e.g., temperature, salinity, nutrients, and pH, as hypothesized in the "hologenome theory of evolution" (11, 66). This theory posits that the microbial associates are intimately tied to their host and therefore changes in host environment can induce shifts in microbial genomes. Thus, the resulting genome is not simply that of the host but constitutes a hologenome, i.e., all the genes of the host and of the members of its associated microbiota (11, 66).

Strong interactions between viruses and bacterial diversity have been previously evidenced under both natural and experimental conditions (67–70). Here, the frequent occurrence of lysogenic and lytic infections in coral mucus (compared with the water column) could then be also partially responsible for the temporal variability of BCS, as shown by the Mantel test and the positive and significant correlation detected between this parameter and the abundance of mucosal viruses in *F. repanda* (Table 4).

The Biolog analyses also revealed distinct carbon fixation profiles between epibiotic and planktonic communities. However, unlike the large temporal variability in the genetic structure of epibiotic communities, their metabolic capacities remained relatively stable throughout the survey; the seasonal fluctuations were even lower than that of their planktonic counterparts. Similar decoupling between the genetic and functional diversity of bacterial communities has been described in numerous other aquatic environments (71–73). Since prokaryotes are highly abundant in coral mucus, genetically highly diverse, capable of dispersion, and physiologically adaptable, they are often assumed to be functionally redundant (74), meaning that different bacterial species could share a same function. In our case, one might also suspect that available substrates types within the mucus are limited and prevent high metabolic diversity and variability. Overall, the nature of the coral mucus may ensure stability of its associated epibiotic bacterial functions, regardless of which bacterial species is providing the functions.

Viral infection of *Symbiodinium*. Viruses of eukaryotes can be also qualitatively important within the coral holobiont, such as those infecting the symbiotic zooxanthella *Symbiodinium* (21). The presence of latent viruses was first detected in these dinoflagellates after exposure to UV, elevated temperature, and even sunscreen products (75, 76). More recently, Correa and colleagues (21), based on the presence of transcribed single-stranded RNA (ssRNA) and dsDNA virus-like genes, showed the first genomic evidence of *Symbiodinium*-infecting viruses in the hard coral *Montastraea cavernosa*. In our study, nearly 400 *Symbiodinium* cells from both coral species were inspected by TEM, without any preinduction treatment. None of the cells showed visible traces of viral infection, indicating that lytic infections were rare or even absent in *Symbiodinium* during the survey. Further investigation will help in understanding the involvement of viruses in the disruption of *Symbiodinium*, their potential impact on the stability of coral-algal symbiosis, and thus long-term reef health and resilience.

Overall, this study provides basic ecological information on the *in situ* abundance and life cycles of viruses associated with scleractinian corals. Coral mucus clearly emerges as a specific habitat favoring viral replication (by both lysogenic and lytic pathways), and the coexistence of these two main reproduction strategies could be a major source of structuration of coral-associated bacterial communities. Further studies are now needed to elucidate how such mechanisms can intrinsically ensure coral stability and fitness to face environmental changes.

ACKNOWLEDGMENTS

This work was financially supported by the EC2CO program and the TOTAL Foundation, with the PATRICIA project. We are thankful to the

French Institute of Research for Development (IRD) for the Ph.D. fellowship grant given to H.N.-K.

We thank the Institute of Oceanography (IO) (Vietnam) and the lab of Ecology of Coastal Marine System (ECOSYM) (France) for their logistic support. We are also grateful to Hue Minh Tran-Thi and Trinh Hai Truong-Si for their participation and technical support, to Hoang Phan-Kim and Quang Thai-Minh for their assistance in diving during the survey in Vietnam, and to Sebastien Villeger for his advice on statistical analysis.

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